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## **HIV-1 SUBTYPE-OPTIMIZED, PERSONALIZED IMMUNOTHERAPY**

Summary of the PhD thesis

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## Introduction

Human immunodeficiency virus (HIV) causes deadly infections worldwide. Currently, 34 million people are living with HIV/AIDS. There is no prophylactic or therapeutic vaccine against the disease on the market, despite of the huge efforts made so far in this research field. The antiretroviral treatment with drugs has several side effects and drug resistant mutant HIV strains are fast emerging, therefore development of an effective vaccine is an urgent unmet medical need.

We are developing a therapeutic nanomedicine and immunotherapy for the treatment of HIV/AIDS. (Nanomedicines are defined by the medical application of nanotechnology for the prevention, treatment and diagnosis of different diseases.)

DermaVir HIV Patch, our first nanomedicine product candidate is already in the phase II clinical investigation stage. The therapeutic use of DermaVir nanomedicine results in de novo generation of HIV specific T cells that are able to kill HIV infected cells in the body of the treated patients. This results in the lowering of the viral load and consequently provides a better immunological state for the treated individuals.

The active ingredient of the nanomedicine is a pathogen-like synthetic nanoparticle consisting of a single plasmid DNA (pDNA) covered by a positively charged synthetic polymer (polyethylenimine-mannose, PEIm) in an aqueous solution. These nanoparticles are administered topically using our DermaPrep medical device to target the nanomedicine to the antigen presenting cells of the lymph nodes (Langerhans cells). T cell specific immune responses are elicited by the 15 subtype-B specific HIV proteins encoded in the pDNA. Multiple safety modifications are inhibiting replicative and integrative functions.

HIV has a large natural diversity represented by 9 subtypes and several circulating recombinant forms (CRF) having 10-35% genetic divergences.

The question raises if the subtype-B specific nanomedicine could result in a proper efficacy in patients infected with this large diversity of HIV strains.

## Aims

We aimed both the *in vitro* and *in silico* characterization of DermaVir nanomedicine. To do this analytical methods were also developed. Our *in silico* T cell epitope predictions revealed the rationale for the establishment of a subtype-optimized nanomedicine product family and personalized HIV immunotherapy. The following tasks were defined to reach the above goals:

- I. *In vitro* characterization of the clinically investigated DermaVir product candidate to prove and support the mechanism of action of the DermaVir immunotherapy.
- II. Development of *in vitro* analytical assays for the investigation of VLP+ expression from the pDNA immunogen of DermaVir and for the quantitative determination of the biological activity (antigen expression capacity) of the nanomedicine.
- III. Development of an *in silico* epitope prediction method for the prediction of the immunological potential elicited by the quasi-proteome encoded in the pDNA.
- IV. Modeling the immunological potential and cross-protection capacity of DermaVir in patients having non-B subtypes of infection.
- V. Design of HIV-1 subtype-optimized pDNA immunogens and development of a design tool for supporting the design and selection of the best-matching product for every patient.
- VI. Cloning and *in silico* characterization of the new DermaVir nanomedicine candidates.
- VII. *In vitro* characterization of the new product candidates.

## Materials and Methods

### Materials and Methods for the nanomedicine characterization

**Nanomedicine preparation.** 1 mg/mL pLWXu1 was diluted with 6 volume equivalent 10% glucose, followed by the addition of 13.6 mM polyethylenimine-mannose (PEIm). Nanoparticle formation was allowed to proceed for 20 minutes at  $23 \pm 2^\circ\text{C}$ .

**Western blot.** 293T cells were transfected with DermaVir and untransfected 293T cells were used as a negative control. Cells were incubated overnight then lysed and frozen in aliquots at  $-70^\circ\text{C}$ . Lysate aliquots were loaded on SDS-PAGE 4-20% precast gradient gel (Bio-Rad) and blotted to PVDF membrane (Bio-Rad). HIV<sup>+</sup> human serum (Boston Biomedica) was used as primary antibody, and anti-human IgG (Sigma-Aldrich) as secondary antibody. Results were visualized by colorimetric detection (Amplified Opti-4CN kit, Bio-Rad).

**Immunoprecipitation.** 293T cells were transfected with DermaVir and with parental wild-type pLW as the positive control. Cells were incubated overnight in DMEM and radiolabeled with 400 microCi  $^{35}\text{S}$  (Perkin Elmer NEG009H) and incubated overnight. Cells were lysed the following day. Lysate aliquots were immunoprecipitated by either inactivated HIV<sup>+</sup> human serum (Advanced BioScience Laboratories) or with monoclonal Nef antibody (NIH AIDS Research and Reference Reagent Program), washed and loaded to 12% SDS-PAGE.

**VLP<sup>+</sup> production and concentration.** 293T cells were transfected with DermaVir. 24 hours post-transfection VLP<sup>+</sup> containing supernatants were collected and filtered by centrifugation through 20% sucrose solution (50,000g, 3h). Pellet was suspended in 1/20 volume PBS (pH 7.4).

**Transmission electron microscopy.** 293T cells were transfected with DermaVir, supernatants were washed with PBS and fixed in 0.1 M cacodylate buffer (pH 7.2) containing 5% glutaraldehyde. The fixed cells were further proceeded by the help of Dr. Attila L. Kovács at ELTE (washing, embedding, sectioning) and investigated in a JEM100CX II electron microscope.

**Potency assay.** 293T cells in DMEM were transfected with DermaVir. After 24 hours, the supernatant of cells was collected and HIV p24 antigen ELISA (Beckman Coulter, alternatively Zeptometrix Retrotek) was performed to quantify the released p24 protein. This assay was validated according to the ICH Q2(R1) guideline [1]. Determination of

the ratio of VLP<sup>+</sup>-associated p24 was performed with modified p24 antigen ELISA assay where detergent was omitted by replacing the lysis and washing buffers with PBS. In general, experiments were performed at least two times with five wells per data point in each experiment.

**Statistical analysis.** To assess the significance of the results, Student's *t*-test was performed on selected data and all *P*-values were calculated.

***In silico* analysis of T cell epitope repertoire.** MHCI and MHCII epitope predictions were made using Immune Epitope Database (IEDB) [2]. We used the Artificial Neural Network (ANN) based prediction method for MHCI and Consensus method for MHCII epitope predictions.

**Phylogenetic analysis.** Sequences from the „Los Alamos HIV Database” were aligned using the Clustal-W alignment method (BioEdit 7.0.9.0). Phylogenetic trees were constructed using Neighbor-joining method and Kimura 2 parameter correction model (MEGA 4.1). Reliability of tree topology was tested by 1000 Bootstrap repetitions.

#### **Materials and methods used for the preparation of pDNA constructs**

**Sequences.** The following subtype-optimized pDNA-s were prepared: pLWXu-AB, pLWXu-BC, pLWXu-C, pLWXu-BF. We used two different sources of HIV sequences for the subtype-optimization: amplified from serum samples of HIV infected individuals (in case of subtype-C, CRF-BC and CRF-BF), or a synthetic gene was designed using sequence from the “Los Alamos HIV Database”. (for CRF-AB). The construct were prepared by replacing the certain parts of the subtype-B specific pLWXu1 pDNA with the subtype-specific ones. All safety modifications of pLWXu1 were maintained.

**RNA extraction.** HIV RNA was extracted from infected serum samples using QIAamp viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions.

**RT-PCR.** Two HIV genome segments were amplified for the constructs: (1) segment spanning the *gag* and partial *pol* genes; (2) genome segment of *env-tat-rev-vpu* genes. Primers were designed based on the sequences of the same subtype with OLIGO 4.0 software. One-step RT-PCR was performed using SuperScript III One-step RT-PCR System kit (Invitrogen).

**Gel extraction.** The RT-PCR reaction samples were run on an agarose gel 0.8% then DNA fragment cut and extracted with “Genelute Gel Extraction Kit” (Omega), according to the manufacturer’s instructions, followed by ethanol-sodium acetate precipitation.

**Ligation and transformation.** pDNA backbone and insert were digested with restriction enzymes, gel extracted and purified as detailed above, then ligated in 1:5 molar ratio overnight at room temperature using T4 DNA ligase (Invitrogen). E.coli chemically competent cells (prepared in house from Stbl2, Invitrogen) were transformed with the ligated pDNA. Transformants were selectively cultured in LB-Kanamycin medium at 30 °C.

**pDNA production.** Suspensions of transformant E. coli were prepared in LB-Kanamycin at 30 °C overnight. pDNA were isolated from harvested bacteria by using E.Z.N.A. Miniprep Kit (BioBasics), or Qiagen Plasmid Maxi Kit-et (QIAGEN) according to the manufacturer’s instructions. After the calculation of the pDNA concentration from the absorbance data measures at 260 nm the final concentration of the pDNA solution was diluted to 1 mg/ml.

**Identity determination of the new pDNAs.** Purified pDNAs were digested with the same restriction enzyme pairs as used for the cloning. Samples containing the expected DNA fragments were sequenced (Eurofins MWG Operon).

## Results

1. We *in vitro* confirmed the expression of the thirteen intact and functional HIV proteins and the two truncated HIV proteins from pLWXu1 immunogen of DermaVir (subtype-B).
2. We confirmed with a newly developed method that HIV antigens expressed from DermaVir assemble to a complex VLP (VLP<sup>+</sup>) in human cells, immunologically authentic to the wild-type HIV. (However, it is safe because it cannot integrate or reverse-transcribe new infectious virions.) VLP<sup>+</sup> is secreted both extracellularly and into intracellular vacuoles.
3. To characterize the antigen expression and to meet the FDA and EMA requirements we have developed a validated potency assay for DermaVir vaccine that characterizes the biological activity of the product candidate by the measuring the quantity of an abundant late structural HIV protein (p24). During the validation we proved that the method is appropriate for the specific, accurate and reproducible quantitative determination of the biological activity of DermaVir.
4. We have developed an *in silico* method for the determination DermaVir's T cell specific immunological potential. The reliability of the selected algorithms for predicting T cell epitopes and the used epitope database were tested by experimentally proven epitopes. We found 97.7% reliability for the MHCI ANN prediction method and 95.1% for the MHCII Consensus prediction method. This means that 97.7% of the 1480 experimentally proven MHCI epitopes were predicted with exact or correct matching (differing only in one amino acid), and 95.1% of the 615 experimentally proven MHCII epitopes were found with exact or correct matching.
5. We predicted the immunological potential of DermaVir with the above methods. We introduced the Absolute immunological potential (AIP) and the Relative Immunological Potential (RIP) for the quantitative and qualitative characterization of the immunological potential. We found that both the early regulatory proteins and the late structural proteins play an important role in the induction of T cell responses with broad specificity.
6. We predicted the T cell specific immunological potential of DermaVir (subtype-B) and modeled its cross-protection capacity in non-B subtype specific infections.

We found only modest cross-protection of DermaVir with other subtype-specific infections of individual patients. This suggests a decreased immunogenicity of subtype-B specific DermaVir vaccine in non-B subtype of infections.

7. We developed a bioinformatics tool for the rational selection/design of vaccine immunogens. Our tool clusters sequences that are producing cross-reactive (identical) epitopes. This tool was then tested during the design of new subtype-optimized nanomedicine product candidates.
8. We prepared four new pDNA immunogen candidates and modeled the immunological potential of them. We found the best cross-reactivity in case of subtype-matching infections.
9. Nanomedicines were prepared from the pDNAs and *in vitro* tested with the newly developed analytical methods. The subtype-B specific nanomedicine served as reference material. We found that all new product candidates are able to express the encoded antigen repertoire (single proteins and VLP<sup>+</sup> too) and the quality of the pDNAs were similar as to the clinically tested DermaVir.



## Conclusions

1. The pDNA immunogen of DermaVir expresses the broadest HIV antigen repertoire constructed for HIV vaccination reported to date.
2. Our *in vitro* results provided explanation to the mechanism of action for DermaVir-derived antigen presentation on the MHCI and MHCII molecules that can be resulted in the induction of both antigen-specific CD4+ and CD8+ T cell responses.
3. Our selected and tested *in silico* methods for T cell epitope predictions might be implemented for supporting the evaluation of clinical immunogenicity data since we can predict the expected T cell responses for each treated patients by considering both the HLA alleles (determining the quality of the immune response) and the sequence of the patient's virus.
4. The novel *in silico* tool developed for immunogen selection/design facilitates the sequence grouping from the immunological aspect (clusters sequences with similar immunological potential) that might be used e.g. for the vaccine development against pathogens with large natural diversity.
5. We suggest the application of the immunogen selection/design tool for the selection of the best matching product and personalized immunotherapy for each patient.
6. Using a subtype-optimized product portfolio and personalized selection of the best matching product by considering the patient's HLA and HIV sequence is expected to result in better immunogenicity results compared to the use of the same immunogen for each patient.
7. Based on the *in vitro* characterization of the four new nanomedicine candidates the quality of them makes the nanomedicines appropriate for the clinical investigation of *in vivo* immunogenicity.

## Publications

### Publications relevant to the thesis

**Somogyi E**, Xu J, Gudics Á, Tóth J, Kovács AL, Lori F, Lisziewicz J. A plasmid DNA immunogen expressing fifteen protein antigens and complex virus-like particles (VLP+) mimicking naturally occurring HIV. *Vaccine* 2011;29(4):744–753. (IF: 3,572)

Tőke ER, Lőrincz O, **Somogyi E**, Lisziewicz J. Rational development of a stable liquid formulation for nanomedicine products. *International Journal of Pharmaceutics* 2010;392(1-2):261-7. (IF: 3,607)

Conference abstract published in a peer reviewed journal:

**Somogyi E**, Gudics Á, Forni DD, Molnár L, Lori F, Lisziewicz J. Subtype-optimized DermaVir for personalized immunotherapy. Abstracts from AIDS Vaccine 2010. *AIDS Research and human retroviruses* 2010, 26(10): A-1-A-184. (IF: 2,082)

### Other publications related to the project

Lőrincz O, Tőke ER, Somogyi E, Horkay F, Chandran P, Douglas JF, Szebeni J, Lisziewicz J. Structure and biological activity of pathogen-like synthetic nanomedicines. *Nanomedicine: NMB* 2011. Nyomtatás alatt. (IF: 4,882)

Patent:

Csörgő SBZ, Garaczi E, Gruber L, Hamar P, Kemény L, Kökény G, Lisziewicz J, Lőrincz O, Molnár M, Mózes M, Ötvös L, Pandur J, Pintér I, **Somogyi E**, Szabó KÁ, Szollár L, Tőke ER. Immunogenic nanomedicine composition and preparation and uses thereof. Pub. No.: WO/2010/125544, International Application No.: PCT/IB2010/051909, Publication Date: 04.11.2010, International Filing Date: 30.04.2010

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[1]ICH Q2(R1) guideline: Validation of analytical procedures: Text and Methodology

[2]Webpage: <http://tools.immuneepitope.org/>